Examining Microalgae species as a Biofuel Energy Crop: A Review

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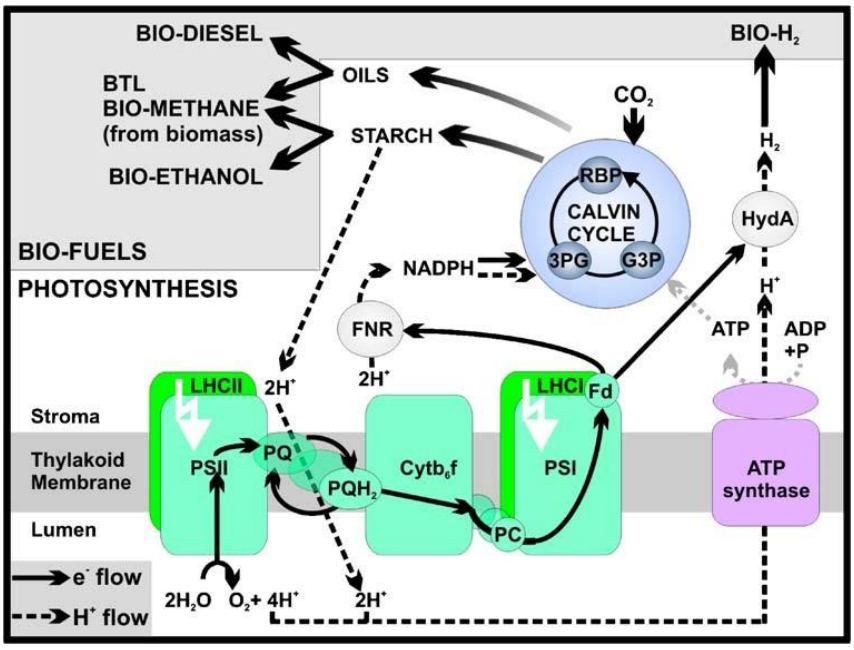
The development of CO2 neutral fuels is one of the most pressing challenges facing our society. In the last 18 months, the Stern Report's "Climate Change Economics" [170] and the National Security Council's "AR4 Synthesis Report" [84] have led to the emergence of this fact. These reports provide the most comprehensive assessment to date of the causes and effects of climate change. Basically, they define atmospheric CO2 levels as above 450 ppm CO2-e (e=equivalent of all greenhouse gases) are currently in the dangerous range and we conclude that we passed the threshold (currently 455 ppm CO2-e) 10 years earlier than the previous estimate. These new discoveries have led the government to set a target (such as the European Union) to reduce CO2, usually in the range of 10-20% by 2020. Germany has recently pledged to cut emissions by 30% by 2020 if other countries do the same.

However, these modest reductions are not sufficient to keep CO2 levels within the accepted "optimal" range (i.e. below 450 ppm CO2-e; IPCC [84]). In fact, they can stay stable above 550 ppm. Damage will be greater at this stage [84]. Therefore, although various CO2 reduction scenarios have been developed to offset emissions, the IPCC concluded that emissions should increase by 2015 and that total CO2 emission reductions should be achieved by 50-85% by 2050. More importantly, the IPCC is an understatement because current climate change models lack carbon feedback. As a result, lower emissions reductions (60% by 2020) are seen as overkill, and this concern prompted IPCC Chairman Rajendra Pachauri to say: "What we do in the next 2 to 3 years will determine our future [126]. However, achieving a 60% reduction in CO2 emissions by 2020 or even a 50-85% reduction by 2050 is a major global challenge that requires a lot of improvement in terms of renewable energy. While biodiesel and bioethanol are considered the closest commercial options, consolidation efforts are underway to produce new biofuels. Biomethane, biomass-to-liquid (BTL) diesel and biohydrogen fields are also developing rapidly. This review provides a brief overview of the background and recent developments in secondary microalgae processes, focusing on biodiesel, which has the potential to be combined with the production of renewable, CO2-neutral gas through the power plant chimney and atmospheric CO2 sequestration. In particular, it aims to analyse the current situation in the biodiesel sector and identify key opportunities for future innovation. Biofuels and Biodiesel in a global context The global energy market can be divided into electricity and oil sectors. Both industries need to achieve reductions in emissions to meet international regulatory targets. Energy currently accounts for about 33% of the world's energy and is evolving in various ways to produce low CO2 energy (eg nuclear, solar, wind, geothermal, hydroelectric, clean coal technology).

By contrast, oil had a larger market share (about 67%) in world electricity consumption in 2005, at about 15.5 TW (489 EJ/year), according to the US Energy Information Administration. However, despite the importance of fuels, CO2 neutral (e.g. biodiesel, bioethanol, biomethane, BTL diesel) and CO2-free fuels, electricity generation has not yet been established. The abundance of biofuels available demonstrates the convenience and potential of the biofuel industry. However, first-generation biofuel systems have not been able to realize this potential due to major economic and environmental constraints (see "Problems in biofuel production" and "Economic feasibility of microalgal biodiesel"). In contrast, second-generation biofuel systems, such as lignocellulosic and microalgae biofuel systems, can overcome many of these limitations and overcome the new clean energy market target of $500 billion by 2050 [170] or more. The majority of biofuels to date are produced by higher plants, which use photosynthesis to convert solar energy into chemical energy (Figure 1). In nature, this chemical energy is stored in various molecules (e.g., lignin, cellulose, starch, oil). Lignocellulose is the main component of plant biomass and can be processed as a raw material for ethanol production. This can be done by gasification or cellulolysis (chemical or biological enzymatic hydrolysis). This process is currently being developed for secondary biofuel systems [41, 155] and is often referred to as "lignocellulosic processes". Similarly, starches (e.g. from maize) and sugar (e.g. from sugarcane) are converted to bioethanol by fermentation [23, 74], while oil (e.g. from sugarcane) is converted to bioethanol. Rapeseed, soybean and palm oil) are used as raw materials for the production of biodiesel [80, 156]. Microalgae can efficiently synthesize cellulose, starch and oil [16, 162]. In addition, some microalgae and cyanobacteria (producing more glycogen than starch) can also produce biohydrogen under anaerobic conditions [20,35,59,76,111] and their fermentation can be used to produce methane.

Fig.1 The process of photosynthesis, which converts solar energy into chemical energy, is essential for all biofuel production in plants. (Schenk et al., 2008)



Flexibility of Biofuel Production Systems:

At the heart of all light-powered biofuel production is the process of photosynthesis. It is the first step in converting light energy into chemical energy and is ultimately responsible for directing the production of raw materials necessary for the synthesis of various fuels: protons and electrons (for biohydrogen), sugars and starches (for bioethanol), fuel (biodiesel) and biomass (for bioethanol).In higher plants and green algae, light is captured by special light-cutti ng proteins called LHCI and LHCII (Figure 1). They are encoded by large gene families that show a high degree of homology [51] and their expression is dependent on the environment (eg light intensity). These proteins bind most of the chlorophyll and carotenoids in plants and are involved in collecting light and dissipating excess energy to halt the photosynthetic reaction, particularly photosystem II (PSII; [81]). The excitation energy used to drive the photosynthetic field is sent to the photosynthetic reaction centre of photosystem I (PSI) and PSII via a highly coordinated pigment network connected by the LHC, PSII and PSI subunits. In a first step, PSII uses this energy to drive the photosynthetic water splitting reaction that converts water into protons, electrons and oxygen. Electrons are transferred to NADPH through the photosynthetic electron transport chain of plastoquinone (PQ), cytochrome b6f (Cyt b6f), photosystem I (PSI), and ferredoxin (Fd) (Fig. 1). Simultaneously, protons are released into the thylakoid lumen via the PSII and PQ/PQH2 cycles. This creates a proton gradient that drives ATP production by ATP synthesis. Protons and electrons recombine with ferredoxin-NADP + oxidoreductase (FNR) to form NADPH. NADPH and ATP are used in the Calvin cycle and other biochemical pathways to produce the sugars, starches, fats and other biomolecules (together as biomass) needed to produce bioethanol, biodiesel, biomethane and BTL biofuels. Alternatively, in some photosynthetic organisms (such as the green alga *Chlamydomonas reinhardtii*), protons and electrons extracted from water (or starch) can be transferred to hydrogenase (HydA) via the ETC.

The Calvin Cycle is an essential part of the photosynthetic process responsible for fixing carbon dioxide in many organisms, from primitive algae to higher plants. The process uses ATP and NAD(P)H produced by light. In C4 and CAM plants, it is combined with continuous processes that lead to CO fixation, but photosynthetic processes reduce the cycle unchanged [175]. The Calvin cycle can be divided into three main steps consisting of carboxylation, reduction and regeneration of the substrate (ribulose 1,5-bisphosphate (RuBP)). The first step in the cycling of CO2 to react with RuBP is catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco). The importance of Rubisco cannot be overemphasized, because the importance of all the carbon monoxide in the world is fixed from atmospheric carbon dioxide in one go by this enzyme. It is also the most abundant protein in the world, accounting for 30% of the total protein in most leaves [129]. This is partly due to its important role in photosynthesis, but also because its catalytic carboxylase activity is very low and uses only 2-3 RuBPs per second [104]. As the name suggests, rubisco has two catalytic functions: it works as a carboxylase, which is part of reducing photosynthesis, and in aerobic conditions, it works as an oxygenase, which is part of photorespiration. O2 and CO2 compete for the same catalytic sites, so the efficiency of CO2 fixation may be affected in some aerobic environments. For example, although the enzyme is highly specific for CO2 (Tobacco plants) 82 times, *Griffithsia monilis* (red algae)

167 times, Rhodospirillum rubrum (sulphur-free blood bacteria) 12 times [7], O2/CO2 molecular ratio in atmosphere 540:1, 24:125° C Air to water is saturated. In the first step of the Calvin cycle, rubisco catalyzes the formation of two molecules of 3-phosphoglycerate from RuBP, CO2 and H2O. Negative changes in the weak process leads to positive effects. In the second step, in the ATP/NADPH-dependent reduction step, carboxylic acids are reduced by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase to produce two molecules of glyceraldehyde-3-phosphate. The third step consists of a reaction in which some of the glyceraldehyde-3-phosphate is converted to RuBP, which is necessary to maintain the reduction of photosynthesis [175].

Addressing problems in biofuel production:
Despite the great potential of the biofuel process to provide a way to produce carbon neutral fuel, the primary production process has many economic and environmental limitations and the topic of biofuels has recently become a hot topic of discussion. While some emphasize the benefits of biofuels [157, 167], others criticize the commercialization and reduction of biofuels [19, 128, 131, 147, 190].

The most common problem with existing biofuel systems is that when there is a large production capacity, they compete with agriculture for arable land used for food production.
For example, current biodiesel production from vegetable oil supplemented with small amounts of animal fat and waste cooking oil is estimated to account for only 0.3% of global fuel consumption (about 12 million tonnes in 2007) [24, 125] available and based on future transportation demands. Currently, about 8% of vegetable oil production is used to make biodiesel [125], which causes the price of vegetable oil to increase over the years.

Area required for biofuel production:
Earth's surface (510,072,000 km2) is estimated to receive an average of ~170 Wm−2 solar energy [31, 196]. This is equivalent to 2,735 YJ of energy per year, equivalent to 5,600 times the world's primary energy consumption in 2005 [196]. Therefore, the solar energy required to produce biofuels is plentiful. But even now oil crops will be planted on all arable land. If 2% of the world is land, 13% is suitable for agriculture, energy conversion efficiency from sunlight to biomass is 1% and oil production is 20%, these will meet half of our energy needs today. Using these figures, biofuel critics often conclude that biofuel production cannot contribute significantly to global oil demand. However, as will be mentioned here, higher photosynthetic efficiency and fuel production have already been achieved, thus paving the way for second generation biofuel technologies with great potential. The increase in arable land has created serious problems and bad practices around the world, giving rise to the term "hill land". For example, rainforests in Brazil and Southeast Asia are being cleared at an unprecedented rate to make room for soybean and palm oil plantations for biodiesel production.

Current biodiesel growth rates in Indonesia, Malaysia and Thailand range from 70% to 250% per year [181]. The increase in the amount of land that can now be used for food production can lead to food insecurity, particularly in countries where more than 800 million people

already face hunger and malnutrition (figures exclude China; [53]). Also, the use of land extensively with fertilizers and pesticides can cause serious environmental problems.

Net energy balance when evaluating the cost and sustainability of the biofuel production process, it is necessary to establish the net energy balance (NEB). Agriculture, harvesting, processing, shipping, etc. Considering the energy required for first-generation biofuels, the NEB is estimated to be about 25% corn ethanol and +∼93% soybean biodiesel [80], but the number depends on the amount of production. detailed Case lifecycle analysis. While this report rejects the claim that the energy cost required to grow crops, machinery and facilities has led to negative NEB values for both biofuels, it has yet to take into account the Intergovernmental Panel on Climate Change's prediction that traditional crops may decline. There will be an increase of up to 50% by 2020 [84]. The carbon balance of the Carbon monoxide calculation process is equally important.

Biofuel crops are generally considered a nearly carbon neutral process because almost all of the carbon in carbon dioxide comes from the carbon dioxide released when burned in situ. But in reality, the overall CO2 balance of biofuel production needs to be evaluated by manufacturer, including energy consumption, plant use and refining, and the process of transporting the fuel from the CO2 that already emits fossil fuels. In addition, oil production from palm oil fields established before the Kyoto emissions target policy is expected to reduce emissions compared to natural gas. Conversely, if the rainforest area has to be cleared first to make room for plantations, the CO2 balance will be negative [14]. CO2 Capture The integration of biofuel production with CO2 separation systems is a significant development that will not only greatly improve the net CO2 balance of the biofuel process, but will also help reduce atmospheric CO2. These usually involve the production of Agrichar via the pyrolysis process.

A number of second-generation biofuel production systems are currently under development that will have more NEBs, use more water and require less arable land. Lignocellulosic technologies and microalgae are of interest ([29,76,96,155]; Table 1). It has been reported that in one region, microalgae produce 15-300 times more biodiesel than crops (Table 1; [29]). In addition, microalgae often have a short cycle (~1-10 days depending on method) compared to traditional crops harvested once or twice a year, allowing them to be grown multiple or sequentially with increased results (Table 1).

Higher light capture and conversion rates ultimately result in less fertilizer and food wastage, reducing waste and pollution. The use of wastewater for algae cultivation is also a viable option [75,103,120]. Also, as microalgae cultivation for biofuel production can be done on marginal or soilless lands, it can reduce competition for land and open up new markets in arid, dry or saline areas. In addition, while biofuel plants always require a lot of fresh water, a large amount of fresh water can be saved if algae cultivation is carried out in low evaporation closed bioreactor systems, especially using marine and halophilic microalgae species. Microalgal biomass produced in bioreactors can also be gasified or pyrolyzed to produce a variety of biofuels and charcoal cultivation as part of CO2 capture strategies. In this way, bio-GMO wastes can be disposed of in a sensitive environment. Carbon-rich biomass pellets can also be

stored as part of a carbon sequestration strategy that uses CO_2 from power plants as feedstock for biomass production. Another important aspect of secondary microalgae systems is their suitability for biotechnological processes that can rapidly restore algae populations. These properties are promising for increasing the "Photosynthetic Efficiency".

Table 1 Comparison of crop-dependent biodiesel production efficiencies from plant oils

Plant source	Biodiesel (L/ha/year)	Area to produce global oil demand (hectares × 10^6)	Area required as percent global land mass	Area as percent global arable land
Cotton	325	15,002	100.7	756.9
Soybean	446	10,932	73.4	551.6
Mustard seed	572	8,524	57.2	430.1
Sunflower	952	5,121	34.4	258.4
Rapeseed/canola	1,190	4,097	27.5	206.7
Jatropha	1,892	2,577	17.3	130 (0[a])
Oil palm	5,950	819	5.5	41.3
Algae (10 g m^{-2} day^{-1} at 30% TAG)	12,000	406	2.7	20.5 (0[a])
Algae (50 g m^{-2} day^{-1} at 50% TAG)	98,500	49	0.3	2.5 (0*)

Presented yields are for peak performing crops [16, 29, 162], although for example, Malaysia's average oil palm yield is actually about 4 tons/ha [119]. Algae yield scenarios are based on existing production systems and their potential [16, 162]. Current algal production systems fall between these ranges: Seambiotic Israel (currently at 20 g m^{-2} day^{-1} at 8–40% Triacylglycerides (TAG), HR BioPetroleum Inc Hawaii (aims to achieve 50 g m^{-2} day^{-1} at 30% TAG)

[a] If algal ponds and bioreactors are situated on non-arable land; jatropha is mainly grown on marginal land.

Recent advances have been made in many areas and examples of improved photosynthetic productivity and growth tolerance in saltwater or wastewater are being determined. Some limitations of secondary microalgae systems have been identified in the harvesting process and CO_2 production efficiency.

Other Biofuels from Microalgae:

In addition to the production of oil for biodiesel production, microalgae is also good as a feedstock for biofuel production. The development of algal biodiesel production technologies is also related to biomass liquefaction (BTL) method using biohydrogen, biogas, bioethanol and fast-growing algae. The BTL, biohydrogen and biomethane processes are discussed below because they are particularly relevant to microalgae systems. Bioethanol is not disclosed here, as the process of producing bioethanol from microalgae is similar to the first- technologies that use food products derived from corn and sugar. Biohydrogen producing Microalgae for photobiological hydrogen production from water have been developed as a promising, potentially emission-free fuel stream that can be combined with atmospheric CO capture. Biohydrogen production by microalgae has been known for over 65 years and was first discovered in the green algae *Scenedesmus obliques* [59] and later in many other photosynthetic species, including cyanobacteria [20, 35]. Most studies of algal hydrogen production used the green alga *Chlamydomonas reinhardtii*, a model organism for photosynthesis studies on the aerobic-anaerobic cycle developed by Melis and colleagues in 2000 [78, 148], and this species was also used in 2000 [63, 111].

The appeal of the biological hydrogen process is that it uses sunlight to convert water into hydrogen and oxygen; this is released in a two-step process. The first reaction occurs in all aerobic photosynthetic organisms, while the second reaction is mediated by specific iron-containing chloroplast hydrogenases and is limited to a more selective group of microalgae [57, 77]. Cyanobacteria can also produce hydrogen from water, but use a different biochemical method. Under normal light and aerobic conditions, H+ and e- produced by photosynthetic hydrolysis reactions are used to combine ATP and NADPH. The second reaction occurs under anaerobic conditions. In the absence of O2, both ATP from oxidative phosphorylation and NADH/NADPH formation are inhibited [67]. In these conditions, some microalgae, such as *Rheinella*, redirect energy stored in carbohydrates such as starch to chloroplast hydrogenases [63, 111] to facilitate ATP production via photophosphorylation and prevent transport chain overload [96, 151]. Therefore, hydrogenases combined with different fermentation processes [93] essentially act as proton/electron release valves to produce hydrogen gas, which is removed from the cell by combining protons and electrons from neutral by reducing ferredoxin [111]. *Chlamydomonas reinhardtii* thus forms the basis of solar-powered biohydrogen from water, and other fermentation processes can also be used. An important advantage of hydrogen production is that hydrogen is not stored in culture but is rapidly released into the gas phase, unlike other fermentation products that can accumulate which are toxic to cells. Recent developments have led to increased efficiency of algal biohydrogen production. For example, strains with increased starch content, decreased cyclic electron flow around PSI (Stm6) and increased external storage of glucose (Stm6glc4) have been shown to improve [42, 95].

To create a profitable commercial algal hydrogen production system, the metabolic flow of hydrogen must be optimized through bioengineering and optimization of related processes in the bioreactor system [76]. Bioengineering includes methods to increase photon conversion from the current ~1% to ~7% commercially available [76]. The "Closed Bioreactor Design" section provides details on actual bioreactor design and ways to reduce costs. Combining hydrogen production with seawater desalination Biohydrogen production from algae can be combined with seawater desalination, even with limited production. Marine and halophilic algae can extract hydrogen (as protons and electrons) and oxygen from seawater, and the hydrogen and oxygen are burned to produce fresh water.

It is therefore possible to combine electricity generation with desalination, using fuel that uses hydrogen and oxygen to generate electricity in the country. Although the freshwater crop is small, it provides freshwater benefits that crops cannot. The effect of water is directly related to the effect of H2. At 1% conversion efficiency of light to hydrogen (approximately the current state of external light using a photodilution generator), research shows that 1 million litres of photobioreactor reactor space can produce up to 610 cubic meters after processing is complete. (610,000 litres of hydrogen) fresh water per year.

Algal Biomethane Production:

Biogas production from biomass has gained importance in the world. A recent study by the Leipzig Energy and Environment Institute estimates that most of Europe's methane demand can be met as biogas [18]. However, current understanding of the biological processes occurring in biogas production facilities is limited. Therefore, research in this area is necessary and important for the improvement of the biomethane process. Biomethane can be produced from different types of biomass and from different plant species. A major limitation for the future development of biomethane-based plants is the availability of photosynthetically grown biomass.

Currently, a 500 kW biomethane plant needs around 10,000-12,000 tons of biomass feedstock per year, and corn is currently the main crop. Typical biomethane yields of between 2,000 and 4,500 m3 per hectare per year have been reported using cereals and sunflowers [5, 192]. Corn yields are high and vary depending on variety and harvest time; produces 5,700 to 12,400 m3 of biomethane per hectare per year [5, 136, 192]. Biomethane production of up to 4,000 m3 per hectare per year has been reported for some grass species such as ryegrass [192].

Microalgae are important for monitoring because biomass production per hectare is about 5-30 times the production of crops [162]. High lipid, starch and protein content and lack of lignin make microalgae ideal candidates for biomethane production by fermentation in biogas plants. Similar to biodiesel, lipids play an important role, since their conversion capacity into biomethane is higher (1390 L biogas (72% CH4, 28% CO2) per kg organic dry substance) than that of proteins (800 L biogas (60% CH4, 40% CO2) per kg organic dry substance) and carbohydrates (746 L biogas (50% CH4, 50% CO2) per kg organic dry substance) [184]. One of the first studies on the possibility of using microalgae to produce biomethane was published about 50 years ago and concluded that the process is possible and could be improved in the future [65]. Microalgae can now be grown in large quantities (150-300 tonnes per hectare per year; using indoor algae bioreactors useful for biomass feedstock and biomethane production. [29, 138]

This amount of biomass can produce 200,000-400,000 m3 of methane per hectare per year. However, it should be noted that biomethane produced by microalgae cannot currently compete with biomethane produced from corn or other crops because biomass is expensive to produce (the commercial value of green algae in Germany, where most biogas producers are located). For example, *Chlorella vulgaris* is 100 times more abundant than maize, and its production capacity is currently too low to meet the needs of biogas plants. Today, methane produced by biogas plants is mixed with carbon dioxide (usually 50-75% methane), whose high impurity content limits its use. Fuel storage is no longer common, as the combustion of biogas is usually done in combined heat and power plants. However, the development of efficient purification systems are already under way and have led to the construction of first pilot plants for biomethane separation in Austria in 2005 (patent no AT411332B (2003)) and in Germany in 2006 (patent no.304 26 097- BCM-method).

Microalgae Cultivation:
Traditional outdoor pond algae production and even some indoor algae bioreactors have been commercially successful in producing valuable products such as astaxanthin and nutraceuticals. Biofuel production systems are less economically viable as they have a lower market price. Therefore, optimized biomass production forms the basis of commercial biofuel production [73,76,100,145,166], which requires careful optimization of crop growing systems.

Optimizing Culture Conditions:
Optimizing strain-specific culture conditions is complex and involves many factors, all of which can create limitations. These include temperature [30], mixing [12], hydrodynamic and hydrodynamic stress [13], bubble size and distribution [14, 135], gas exchange [52], mass transfer [68], water quality, pH, salinity [1,30,140,141], mineral and carbon regulation/bioavailability, cell fragility [70], cell density and growth inhibition [16]. While controlled agitation of the culture can provide important preliminary information, appropriate models must be properly constructed in laboratory bioreactors [150] to ensure mixing and mass transfer. Traditionally, most research on the relationship between algae growth and nutrition has been devoted to algae in the natural environment and the role of algae growth in ecosystems.

In contrast, algae production systems require maximum biomass growth to achieve high cell densities. Better understanding of physical principles and bioreactor design [66] has increased available cell densities. Optimum media formulation is also important to provide adequate and stable nutrients for maximum and rapid cell growth and ultimately higher efficiency biofuel production [36, 37]. Algae production can also be a multi-step process with great freedom for each step, such as nitrogen limitation in oil production [165] or sulphur limitation in hydrogen production [111]. Fed batch feeding of heterotrophic algae culture [100] and enrichment of photoautotrophic algae culture with CO2 can increase biomass, while optimization of mineral nutrients can increase productivity of insect traditions. Nitrogen and phosphorus are often the first targets of improving the mineral environment [28,86,165,203], but other minerals are also important to support the processes and metabolic biochemistry of cells. Mineral ions also have important effects on areas such as osmoregulation and osmotic adaptation [89, 90] and the molecular configuration of photosynthetic complexes [112].

Some types of algae grow twice as fast and have twice the number of cells when grown with yeast extract. Growing bacteria are apparently able to assimilate dissolved organic molecules, so in some cases wastewater can be considered a resource. The scarcity of fresh water resources in many countries also leads to the recycling of wastewater, and studies continue on different ways of using wastewater [10,153,154,163,201]. Zaslavskaia et al. [202] demonstrated the transformation of *Phaeodactylum tricornutum* (a functional photoautotroph) into a heterotroph through genetic modification of the same gene encoding the glucose transporter.

The identification, characterization and engineering of carriers shows great potential for future use in algae culture, particularly in biofuel production. Controlling the pH achieved throughout the culture is important because it affects all aspects of the biochemistry of the medium. The

ion uptake in the medium and the metabolic biochemistry of the cells can cause significant stress on pH, and in high-activity cultures their effect is sufficient to overcome the potential for the absence of exogenous buffers. Currently, microinjection of strong acids and bases, stabilization in heterotrophic cultures, and control of CO_2 dissolution in photoautotrophic and heterotrophic cultures [52, 173] are among the best practice and business for pH control.

Outdoor Pond Systems:

Most of the microalgae grown today are grown in open ponds. The construction and operation of open ponds is very efficient and therefore more efficient as long as the culture is preserved [193]. Outdoor pools can come in many shapes and sizes, but the most common design is a racetrack pool. Divide an area into a grid of rectangles, each containing an elliptical channel; The impeller is used to maintain a constant flow of water in the circuit. They usually work at a water depth of 15-20 cm because at this depth the biomass concentration is 1 g dry weight per litre and the productivity is 60-100 mg L-1 day-1 (eg.10–25 g m−2 days−1) is possible [137]. However, this efficiency is not always available and cannot be sustained on an annual average. Similar designs are also found in reservoirs in Asia and Ukraine [15]. Algae pools used in wastewater treatment plants can be designed to best adapt to the site; they are usually not mixed, but guided by gravity flow. One of the largest of its kind is the Werribee wastewater treatment plant in Melbourne, covering 11,000 hectares.

For algal wastewater treatment ponds, retaining walls or dug trenches form the basis of the pond. Dams are more expensive to build due to the additional mechanisms (vanes) required, and higher flow rates mean more stable structures are required to maintain the stability of the lake. However, since transparent materials are not needed to create an open-air pool, various materials can be used for construction. Outdoor pools are also easier to maintain because they have wide open channels that remove surface biofilm. The biggest disadvantage of the open system is that they lose water by evaporation at the same rate as the products in the soil when they are open to the air, and they are also susceptible to harmful diseases. Newly opened ponds are often inoculated with the desired algae culture to encourage algae growth and preserve the pond's flora. However, undesirable species will occasionally appear and can significantly reduce yields even beyond inoculated species. When a big competitor enters the pool, it is very difficult to get rid of it. Of the more than 3,000 photosynthetic organisms collected by the Aquatic Species Program, none were found to dominate the open ponds and have the necessary biofuel and high lipid content [162]. In practice, it has been reported that open ponds are usually of two to six types and have many advantages: rapid growth, protection against animals, tolerance to high oxygen levels, etc. Continuous and reliable breeding of the same species in open ponds However, the improvement of systems can be supplemented by breeding extremophiles that avoid and outperform other species in certain environments (eg high/low pH or salinity). *Spirulina*, for example, survives and grows well at high pH (9 to 11.5) and is often the dominant species in soda lakes [17]. It is also easy to collect due to its spiral shape. Australia is the world's largest producer of *Dunaliella*. This unicellular type of green algae grows well in high salt water due to its high intracellular glycerol content, which protects against osmotic stress. *Dunaliella salina* is the best source of beneficial carotenoids that protect itself from harsh light in shallow lakes [21].

Closed Bioreactor Design:

Closed bioreactors have many other advantages besides water, energy and chemical savings and are increasingly becoming the reactor of choice for biofuel production as costs fall (Fig. 3). The most important thing about these products is that they support up to five times more production in terms of reactor volume and therefore have a smaller "footprint" in terms of production [13]. The second point is decision, because the goal is to collect as much solar energy as possible from a piece of land. Therefore, as recent studies have shown, production costs can offset higher bioreactor costs [29]. Most closed photobioreactors are designed as tube reactors, plate reactors or bubble column reactors ([137, 193]; Figures 3 and 4). Other less complex structures, such as semi-hollow spheres, have also been reported to be effective [152].

However, there is still a gap between the design of high-end reactors that meet all the needs of algae cells on the one hand, and the design of inexpensive reactors, on the other hand, to improve process economy [193]. Based on current electricity prices and production costs, the cost per square meter of the reactor should not exceed $15. The following paragraphs discuss some aspects of reactor design and current development from a business perspective. Most microalgae exhibit growth/photodynamics where light saturation occurs at medium light intensities. Therefore, they have low activity, photoinhibition, and even photobleaching under direct sunlight for *Chlamydomonas*; [110, 134]. To increase the efficiency of the process, photobioreactors must be designed to scatter light over a large area to provide moderate light intensity to cells. This is usually achieved by arranging tubular reactors in a fence-like structure (Figs. 3) The fence faces north/south to avoid blindness from the field. This elegance is way sunlight is diluted both horizontally and vertically. Dry weight up to 47 g m-2 day-1 can be obtained using this system [27].

The distribution of light around the circle of the tube is very interesting considering the reflections. However, compared to other biochemical engineering processes, this is often not achieved by precise measurements and kinetic methods. To improve illumination, the bioreactor surface can be ten times larger than the corresponding covering area. Similar explanations can be made for photobioreactors with bubble columns or plates placed at an angle to the sun. Both types of structures help improve cell growth but require more transparent materials such as glass or plastic. But the design principle is to "make the ratio of surface area to volume as large as possible". The 400 m2/m3 value is state of the art and such structures are built along a short optical path and thus support higher biomass concentrations. Therefore, small culture volume and less mixing power are required for a given biomass yield. Stirring is necessary in all photobioreactors because it inhibits cell aggregation and promotes CO2 and O2 distribution [117]. The partial pressure for CO2 is at least 0. To avoid limitation of kinetic CO2 uptake, 15 kPa must be maintained and meet the stoichiometric requirement of 1.7 g of CO2 per gram of biomass. That makes supply with CO2-purified from external flue gas e.g. from a power plant useful [45]. Although the light attenuation in the reactor is not affected by the mixture, there is a relationship between the culture mixture and the light attenuation as each algal cell passes through the darkness and light of the reactor more or less interacting [13]. The dark areas are due to mutual shadowing of cells on the side of the reactor furthest from the light source. Therefore, it is necessary to reconsider algae physiology in the context of the so-called

"flashing light effect " [69]. Although high light intensity stimulates the photosystem, it also causes photoinhibition. Therefore, microalgae have evolved their photoprotection mechanisms that removes excess energy in the form of fluorescence and heat. This waste of energy is avoided when the algae cells are mixed and subjected to low/high light cycles because low light allows the energy in the photosystem to be transferred to the metabolic process. The frequency of these cycles should be 10 Hz or faster, and the dark cycle should be ten times longer than the light cycle [85]. Algal cells then behave similarly under constant light conditions [199]. In photobioreactors, flashing light effect can be completely captured with smart mixing to optimize the transition of cells from dark to light and vice versa. This idea has already been implemented as shown in Fig.4 [109]. The reactor is a plastic plate, air-lift reactor with built-in baffles. These cause a constant horizontal vortex of the liquid. But the disadvantage here is the use of the power of mixing technology. However, such reactors have been used successfully. The first CO2 separation demonstration plant will be built in Hamburg, Germany, in 2008. In many different reactors, combined energy is considered to be 10-30% of the light energy problem. This is obviously too much because it exceeds the value of energy.

Plastic bags (polyethylene) are assembled as annular reactors (to prevent darkness in the cylinder) or plate reactors are sold [146, 179]. It turns out that a simple plastic pipe installed horizontally on the ground is not strong enough. One of the latest developments is the triangular reactor (Fig. 5). It combines the principle of a bubble column with mixing by in-built static mixers in an external ‘downcomer’. According to the MIT press release and external evaluation [139], even under the best lighting conditions, this "3DMS-Reactor" exhibits a dry air mass of 98 g m-2 days -1 in a 19-day period. As a result, this is one of the best cultures ever produced, with a theoretical maximum average of about 100 g m−2 days−1. Further enhancements now need to be made using thin layers with high internal area for light distribution. These increase the biomass concentration and reduce the mixing energy [150]. In addition, the transport of the gas should be done by transmission alone, negative energy foam should not be formed.

Another bioreactor design involves collecting light through plastic Fresnel lenses and directing it into lump reactor with optical fibers Separating the infrared portion of the sun's rays not only reduces overheating problems during high solar irradiance, but also allows the ejected radiation to be used to generate electricity. This can be used to mix cultures in the currently most energy-intensive bioreactors. This principle eliminates many of the problems associated with large outdoor reactors, but it is very expensive to use and the technology needs to be further developed to reduce its cost and ensure it is successful. Figure 6 presents a schematic of four commonly used bioreactor designs.

In conclusion, photobioreactor design, especially for biofuel production, is a rapidly expanding field that is important for the rapid development of second generation microalgal biofuel systems. Only new designs combined with knowledge and theory on microalgae dynamics and growth efficiency will lead to optimum and commercial results.

Fig. 3 A high-end closed bioreactor system. The world's largest closed photo-bioreactor in Klötze, near Wolfsburg, Germany (Bioprodukte Prof. Steinberg; www.algomed.de); the 700 m^3 are distributed in 500 km of tubes and produce up to 100 t algae biomass per year

Fig. 4 Flat panel airlift from Subitec [38, 102] with usage of the flashing light effect, here for astaxanthin production

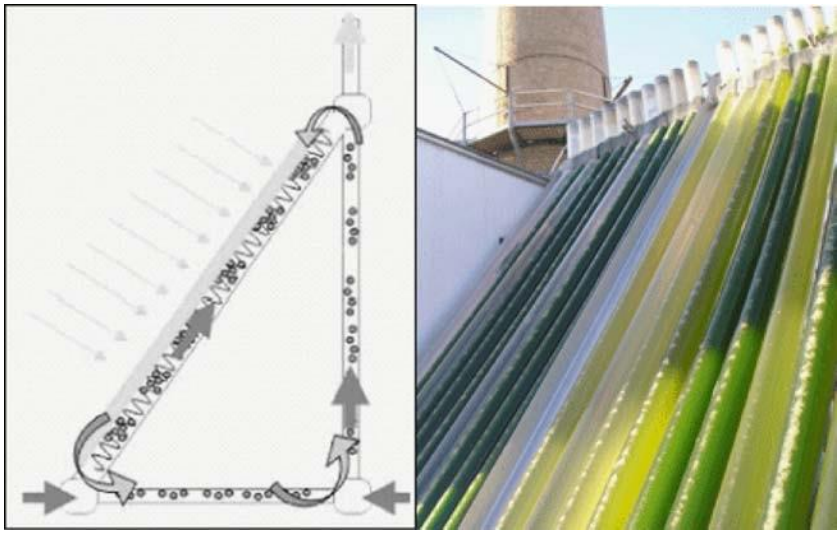


Fig. 5 GreenFuel's 3D Matrix Algae Growth Engineering Scale Unit, "triangle airlift reactor". At the left there is the drawing from patent US 20050260553, at the right the demonstration plant at the Red Hawk Power Plant, Arizona, USA [50]

Hybrid Systems:

Outdoor ponds are a good and economical way to grow algae, but they can become infected quickly. Photobioreactors are excellent for maintaining sterile cultures, but setup costs are often ten times higher than outdoor pools. The combination of the two systems would be the best option for the efficient cultivation of crops useful for biofuels. Inoculation has long been a part of algae cultivation. Open ponds are inoculated with a desired strain that was invariably cultivated in a bioreactor, whether it be as simple as a plastic bag or a high-tech fibre optic bioreactor. Importantly, the size of the inoculum needs to be large enough for the desired species to establish in the open system before an unwanted species. But sooner or later contamination dominate the system and it will have to be cleaned and re-inoculated.

Therefore, cleaning or washing the pools should be a part of the aquaculture process to reduce the problem. Aquasearch (Hawaii, USA) demonstrated this process using *Haematococcus pluvialis* to produce astaxanthin. Half of the Aquasearch area is dedicated to photobioreactors and the other half to open ponds. *Haematococcus pluvialis* was grown continuously in a photobioreactor in a nutrient-rich environment, and then some of it was transferred to a nutrient-restricted outdoor pond to stimulate astaxanthin production. Enough nutrients are transferred with the inoculum for the culture to continue to grow for 1 day, and after 3 days when astaxanthin level peak, the open ponds are harvested, cleaned and then re-inoculated [83]. This approach is also very suitable for biofuel production as under low-nutrient conditions algae rapidly start to convert energy from the sun into chemical energy stored as lipids as a means of survival (see “Lipid production by microalgae in nature” section). For large scale microalgae biofuel production there would need to be a series of photobioreactors of increasing size, from starter culture through to the final inoculum. As the bioreactors increase in size, the level of complexity should be reduced to minimize the cost per square meter. Smaller bioreactors need to be kept strictly under axenic conditions but as the bioreactor size increases the level of containment can be relaxed if there is a continual resupply of inoculum to flush each stage of the scale up, provided there are protocols in place to eradicate contamination if it takes hold early in the scale up chain. For such an approach to work, it is important to use an algal species that is both, fast growing during the inoculum scale-up stage and highly productive in the final open pond stage. This process allows a continual supply of fresh inoculum into open ponds at environmentally-friendly low nutrient conditions to avoid the dominance of invading species while encouraging the continuous production of algal biofuels.

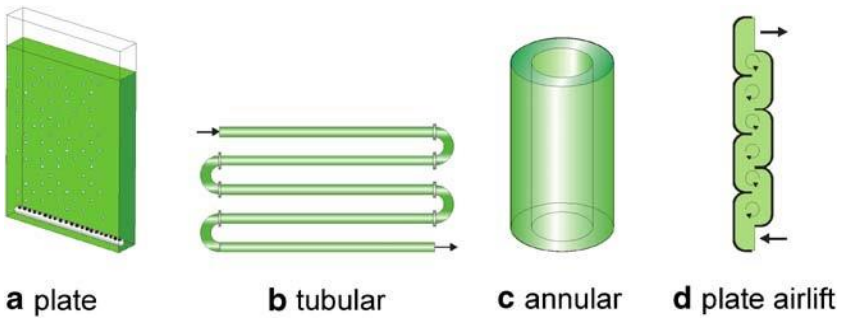


Fig.6 Different closed photobioreactor designs commonly employed for production of valuable compounds: a plate reactor, the classical approach, b tubular reactor, biggest closed photobioreactor is made in this design, c annular reactor, acts as bubble column, the inner cylinder is empty to avoid dark parts and to increase surface/volume ratio, d plate airlift reactor with baffles supports flashing light effect by controlled fluid barrels. All these designs are regarded as in general being suitable but also being too expensive for biodiesel production

Downstream Processing Harvesting Methods:

Algae typically have a high-water content and downstream harvesting and processing requires its removal. There is no single best method for harvesting microalgae and reducing their water content. The most common harvesting processes existing techniques of algal aquaculture are flocculation, microscreening and centrifugation. More energy-efficient and cost-effective harvesting methods need to be devised to make the biofuel production process more economical. In this regard, strain selection is an important consideration since certain species are much easier to harvest than others. For example, the cyanobacterium Spirulina's long spiral shape naturally lends itself to the relatively cost- and energy-efficient microscreen harvesting method [11, 16]. Cost-effective filtration, however, is limited to filamentous or large colonial microalgae. While filtration is often applied at a laboratory scale, in large-scale applications it suffers from problems such as membrane-clogging, the formation of compressible filter cakes and in particular, from high maintenance costs. Sedimentation and centrifugation can be described by Stokes' law, which predicts that the sedimentation velocity is proportional to the difference in density between the cell and medium on the one hand and on the square of the radius of the cells (Stokes radius) on the other hand. While for bacteria gravitational force-based methods are not easy to apply, for yeast and microalgae with diameters >5 μm and relatively thick cell walls they are feasible. Pure sedimentation is employed in some algal farms, but it requires a lot of time and space and is not a good choice for biodiesel production. Commercial centrifuges accelerating to at least 10,000 × g enhance separation and decanting centrifuges have also been successfully employed (e.g. [195]). Currently, centrifugation is considered to be too cost- and energy intensive for the primary harvesting of microalgae. The energy input alone has been estimated at 3,000 kWh/ton [16]. Centrifugation is however a very useful secondary harvesting method to concentrate an initial slurry (10–20 g/L) to an algal paste (100–200 g/L) and could possibly be used in combination with oil extraction. Flocculation, the aggregation and sedimentation (or floatation) of algal biomass, is also a very common primary harvesting method used to concentrate algae [118]. In raceway or mixing ponds, adjacent settling ponds are used for flocculation, settling and harvesting. Inorganic chemicals such as alum, ferric chloride and lime are very effective flocculants but are considered to be too expensive for largescale operations. Organic cationic polyelectrolyte flocculants like the cationic polymer Chitosan are usually preferred as they are required much and the algae can be used in downstream downstream applications, such as animal feed supply or for anaerobic digestion [118]. Flocculation increases particle size and leads therefore to faster sedimentation (see Stokes' law) or better, interaction with floatation bubbles. Nevertheless, addition of flocculants is currently not a method of choice for cheap and sustainable production. Recent developments involve encouraging self-flocculation of the

cells, which can occur during carbon limitation or pH shifts. Polyelectrolytes can also be used to assist spontaneous flocculation (bioflocculation). Bioflocculation is likely the cheapest harvesting process. Certain species naturally flocculate, while others flocculate in response to environmental stimuli, nitrogen stress, pH and level of dissolved oxygen [16]. The problem however with spontaneous flocculation is the time it can take to occur which is not always reliable. An interesting variation of this, recently presented by Ami Ben-Amotz, is co-bio-flocculation [3]. Here the naturally flocculating alga *Skeletonema* was used to form flocs with high lipid varieties of *Nannochloropsis*. Another interesting method recently presented by Mike Massingill, is to feed the algae to the fish Tilapia (*O. mosambicus*). The algal biomass is then harvested from the sedimented droppings by a conveyor belt (10–14% solids) and then air-dried ([3]; Kent Seatech: United States Patent 6447681). Lipid Extraction and Transesterification The majority of biodiesel today is produced from animal or plant oils through a transesterification process following oil extraction with or without cell disruption. Alternatively, the process can be facilitated by combining the use of immobilized lipases with methyl esterification [100]. In essence, a common extraction process involves mechanical crushing followed by squeezing. While cell disruption can be carried out by high-pressure homogenization ("French press") a modern approach is electroporation, where a high electrical field is applied to the biomass leading to perforation of the cell wall and to better extraction. For extraction of oils and other microalgal products, chemical solvents can be chosen in one- or twostep extraction approaches. This can even be applied to living algae in situ [79] or combined with transesterification using methanol and a catalyst such as sodium methoxide to produce biodiesel and glycerol [58].

CO2 Sequestration:

Atmospheric CO2 levels have already exceeded 450 ppm CO2-e and are at levels classified as "dangerously high" [84, 170]. So, although the development of CO2-neutral biofuel production systems is important, their production will largely serve to stabilize atmospheric CO2 levels at a "dangerously high" level, and not reducing it to an acceptable concentration. Physical sequestration of atmospheric CO2 is often considered challenging as it is technically difficult to separate CO2 from other atmospheric gases. Photosynthetic organisms have however fine-tuned this process over millions of years and of course are well adapted to capturing CO2 and storing it as biomass. If this captured CO2 could therefore be converted to a more stable form for long term storage (∼100 years or more) it would open up the important opportunity to couple CO2-neutral biofuel production (e.g. biodiesel) with atmospheric CO2 sequestration. Weissman and Tillett [194] studied the capture of carbon dioxide by large pond-type systems. When operating under optimum conditions, the capture efficiency has been shown to be as high as 99% [194, 204]. Based on the following equation, 1.57 g of CO2 is required to produce of 1 g of glucose: 6CO2 þ 12 H2O þ light þ chlorophyll ¼ C6H12O6 þ 6 O2 þ 6H2O Kurano et al. [97] reported fixation of 4 g CO2 L−1 day−1 at growth rates of 2.5 g algae L−1 day-1, a ratio of 1.6 to 1. Taking into account the conversion of glucose into other compounds such as lipids or starch under certain conditions; the consumption of CO2 can be as high as 2 g CO2 to 1 g algae. Assuming a growth rate of 50 g m-2 day−1 it is possible for one hectare of algal ponds to sequester up to one ton of CO2 a day. In the case of the biodiesel process, after extraction of oil (e.g. ∼30% of the dry weight), the remaining 70% biomass can be fed into

downstream carbon sequestration processes. Specifically the sequestered carbon can be converted to hard C-chips (Agri-char) via the 'slow burning' process of pyrolysis [25]. This has the additional advantage that Agrichar, as its name suggest, can be marketed to the agricultural sector, as it greatly enhances the carbon content of the soil and so its fertility. This in turn can contribute further to climate change mitigation by affecting the gas exchange of crops and soils [98, 105]. Furthermore, pyrolysis acts as a sterilization process of the biomass waste, providing an environmentally-sensitive waste disposal mechanism that will increasing the public acceptance of the use of genetically modified microalgae for biofuel production.

Molecular Improvements of Microalgae for Increased Biodiesel Yields:

The following can be kept in mind so as to obtain the best performing microalgae strains for maximal biofuel production:

(1) screening a wide range of natural isolates,

(2) improve them by metabolic (genetic) engineering or

(3) improve them by selection and adaptation.

Algae collections worldwide contain thousands of different algal strains that can be accessed, for example information about available cultures of algae strains in Europe can be obtained via the Algi-Net Database [4]. Notably, the US Aquatic Species Program had collected 3000 algal strains and assessed these for potentials of biofuel production [162]. Global algae collections and species maintained from the Aquatic Species Program combined with recent advances in genetic engineering and material sciences, provide a good starting point for further development of microalgal biodiesel production systems. Methods like lipidomics, genomics, proteomics, and metabolomics can be used in the future to screen for and develop new strains that show high growth, high rates of lipid biosynthesis, broad environmental tolerances, and produce by-products of high value. Metabolic Engineering and Systems Biology Approaches Bottle necks in algal biodiesel production within the cell can be identified by transcriptomics, proteomics and metabolomics approaches. In contrast to a pure genomics approach the integrated use of these methods allows insight into cellular processes. The identification of differentially expressed genes, proteins or metabolites gives clues to rate-limiting processes in the cell, which can be backed up by the determination of metabolic flux. This systems biology approach will allow fine-tuning of algal properties by genetic or metabolic engineering.

Metabolomics aims to determine metabolic profiles to define the metabolome of a given organism [56], in this case lipid-rich algae. It is a statistical method used to identify the differences in metabolite levels due to either genetic or environmental changes. The techniques commonly used to assess this are NMR spectroscopy or mass spectrometry (in combination with various chromatography techniques) followed by chemometric analysis [101, 123, 124]. The interpretation of these results alone is not straightforward as the accumulation can be due to an up-regulated enzyme downstream, or a downregulated enzyme upstream, in the metabolic pathway. Therefore, this data should be interpreted in context with transcriptomics and proteomics results [121]. To resolve the metabolic dynamics of microalgae, the metabolic flux can be studied by various techniques such as the monitoring of consumption and production of key compounds [44, 197], or the isotopic labelling of key metabolite precursors or intermediates and the monitoring of these isotopes in a time-dependent manner [55].

Radioactive isotopes [34, 158, 198] or stable isotopes for NMR spectroscopy [159, 169] and mass spectrometry [158, 161, 185, 198] can be used to obtain a time- and in some cases spatially-resolved picture of the metabolic flux of an isotope from a given starting compound to a metabolic end product. Transcriptomics and proteomics offer the additional possibility of identifying differentially expressed genes and proteins that are either directly involved in lipid biosynthesis and degradation or that are co-ordinately regulated. For example, the identification of key regulatory genes and their proteins, such as transcription factors, kinases and phosphatases, and their over- or under-expression in transgenic cells can efficiently alter whole physiological pathways [6, 43, 107]. Fatty acid production and composition has been altered in a number of plants by metabolic engineering using transgenes encoding for different enzymatic steps in fatty acid biosynthesis/modification pathways, most notably in canola [39, 49].

After identification of the pathways and key enzymes involved, genetic engineering has the potential to improve algal productivity. Routine transformation is currently carried out only for a few selected algal model species including *C. reinhardtii*, however, the growing field of transgenic microalgae has considerable potential [99, 189]. Different transformation methods are available for the delivery of DNA into the algal genome with the 'biolistic' technique being the most common one [189]. This technique involves bombardment with DNA-coated microprojectiles and has been successfully used for a variety of algae including green algae and diatoms [8, 94], and is also the method of choice for chloroplast or mitochondrial genome transformation [143]. Other methods to create transgenic algae are the agitation of cells in the presence of glass beads and DNA [88], agitation with silicon-carbide whiskers [46, 47], and electroporation [164, 174]. More recent developments for the improved overexpression of transgenes, involve the use of vectors containing nuclear matrix attachment regions (MARs) to increase the expression level of foreign genes. This has been carried out in the halotolerant algae *Dunaliella salina* [191]. Using the glass bead technique of Kindle [88], Doebbe et al. [42] sccessfully introduced the HUP1 (hexose uptake protein) hexose symporter from *Chlorella kessleri* into the mutant strain, *C. reinhardtii* Stm6. This resulted in an engineered mutant that can use externally supplied glucose for hydrogen production. Another starting point for the metabolic and genetic engineering of microalgae is the engineering of the photosynthetic light capture machinery in order to improve solar energy to biomass conversion [122]. The genome of *C. reinhardtii* has recently been sequenced and revealed among others, previously unknown genes associated with photosynthetic functions [113]. Electron pathway optimization can also be considered with the aim of increasing PQ levels by genetically modifying parts of the PQ pathway to decrease photodamage and increase photosynthetic efficiency under high light conditions, and therefore, to increase the biomass production rate [122]. The biosynthesis of algal lipids requires acetyl-CoA as the starting point. Acetyl CoA carboxylase and other enzymes of the lipid biosynthesis pathway have been used as targets for improving oil production [142, 162]. Lipid metabolism, and the biosynthesis of fatty acids, glycerolipids, sterols, hydrocarbons and ether lipids in eukaryotic algae have been recently reviewed in the context of optimization for biodiesel production [72, 115]. While *C. reinhardtii* serves as a model organism to study lipid biosynthesis in green algae [205], some unusual hydrocarbons and ether lipids from *Botryococcus braunii* have been described (e.g. n-

alkadienes, trienes, triterpenoid botryococcenes, methylated squalenes, tetraterpenoids; lycopadiene; [2, 115]. The type of oil used as a biodiesel feedstock has a large impact on the quality of the fuel product. Genetic engineering of key enzymes in specific fatty acid production pathways within lipid biosynthesis is therefore a promising target for the improvement of both quantity and quality (chain length and saturation grade) of lipids. Lipid quality is an important issue for biodiesel production, as the alkyl ester content dictates the stability and performance of the fuel, and this in the end is an important factor in meeting international fuel standards. The volumetric energy density of biodiesel is about 33 MJ/L, which is about 92% of petro-diesel and so essentially comparable. However, the average hydrocarbon length is longer in biodiesel, resulting in combustion that is hotter and sustained for longer than in conventional diesel fuel. A more complete burn of the fuel is achieved. When taking this into consideration the overall efficiency of biodiesel is approximately 97% of that of petro-diesel [92]. Biodiesel is currently produced from a number of different oilseed crops, most commonly soybean, rapeseed and palm oil. As the triacyl glyceride (TAG) profile of a given biofuel crop is relatively consistent, the property of biodiesel produced from a particular crop has predictable qualities. There are a number of factors that must be considered when developing a new source of TAGs into biodiesel. Microalgal lipids are predominantly polyunsaturated, and so are more prone to oxidation. This is a serious issue with biodiesel whilst in storage. This drawback, however, can be corrected through partial catalytic hydrogenation of the oil [29]. Gunstone and Hilditch [71] measured the relative rate of oxidation for the methyl esters of oleic (18:1), linoleic (18:2), and linolenic (18:3) acids to be 1:12:25. Therefore, keeping the levels of polyunsaturated fatty acids in biodiesel minimum is preferable. In contrast, higher levels of polyunsaturated fats lower the cold filter plugging point (CFPP); the temperature at which the fuel starts to form crystals/solidifies and blocks the fuel filters of an engine. Table 2 presents the melting point of the major fatty acids. It is observed that the melting point is lower, more unsaturated an oil is. Therefore, colder climates require a higher unsaturated lipid content to enable the fuel to perform at low temperatures. Cetane number (Table 2) is another measure describing the combustion quality of diesel fuel during compression ignition. In certain diesel engines it is observed that higher cetane fuels have shorter ignition delay periods as compared to lower cetane fuels. Hence, it is important to ensure that the cetane number of biodiesel meets the engine cetane rating [91]. With these considerations in mind, the “ideal mix” of fatty acids has been suggested to be 16:1, 18:1 and 14:0 in the ratio 5:4:1. This type of biodiesel would retaining a good CFPP rating and cetane number and yet have the properties of very low oxidative potential. Algae have excellent potential for the genetic modification of their lipid pathways; e.g. by up-regulation of fatty acid biosynthesis or by downregulation of β-oxidation. By knocking out or modifying enzymes responsible for the synthesis of polyunsaturated lipids in the cell, it should be possible to dramatically increase the proportion of monounsaturated lipids. Additionally, it is likely that the algal cells homeostasis mechanism would have to modify the lipid ratio to remain fluid at low temperatures by decreasing its levels of saturated lipids.

The lipid profile of an algal species will remain consistent provided it is grown under the same conditions. However, every algal species will have its own lipid profile and it is therefore important to utilize species that have a suitable lipid profile for biodiesel production.

Photosynthetic Efficiency:

Apart from metabolic engineering approaches to increase lipid production, two other examples are briefly described below that have potential usefulness for large-scale cultivation: the increase of photosynthetic efficiency, as well as the selection and improvement of strains for optimal growth, survivability, and oil production using wastewater and seawater resources. Any increase in photosynthetic efficiency will enhance downstream biofuel production. Photosynthesis drives the first stage of all biofuel production processes (Fig. 1). Specifically, it captures solar energy and stores it as chemical energy (e.g. oil, starch). Consequently, increasing the light capture efficiency is a significant innovation in the development of all second-generation biofuel production systems. Most wild-type microalgae have evolved genetic strategies to assemble large light-harvesting antenna complexes which capture sunlight and transfer the derived energy to PSI and PSII to drive the photosynthetic reactions (Fig. 1). In nature, the advantage of this strategy is that it maximizes light capture under low light conditions. However, the downside is that as excess light damages the photosynthetic machinery, higher plants and algae had to evolve photo-protective mechanisms [82, 130]. These typically dissipate (i.e. in the context of biofuel production, 'waste') most of the captured energy as fluorescence and heat [134]. This energy dissipation takes place largely in the light harvesting complexes associated with PSII (i.e. LHCII in Fig. 1). It has been demonstrated that the overall light conversion efficiency of bioreactors can be markedly improved by reducing the number of the chlorophyll-binding LHC proteins in each cell [122, 134]. This strategy can be used to carefully fine-tune and optimize light capture efficiency of the antenna systems specifically for oil production. Since wild-type algae have large chlorophyll-binding LHCII systems, the culture is dark green. Cell lines with small LHCII antenna systems yield cultures which are a much lighter green at the same cell density (Fig. 7a). In the wild-type case, algal cells at the illuminated surface of the bioreactor that are exposed to high light levels capture the bulk of the light, but waste up to ~90% of the energy as fluorescence and heat [122, 134]. The further the wild-type cells are from the illuminated surface they are exposed to decreasing levels of light. These shaded cells are prevented from capturing enough solar energy to drive photosynthesis efficiently. As a result, the efficiency of the overall culture is drastically reduced. In contrast, small antenna cell lines with reduced LHCII levels have the advantage that they improve the light penetration into the bioreactor (Fig. 7a) and better match it to the energy requirements of each photosynthesizing cell. Thus 'small antenna' cells at the bioreactor surface absorb only the light that they need, largely eliminating fluorescence of excess energy. This in turn allows more light, i.e. the light wasted in wild-type as fluorescence and heat, to penetrate into the bioreactor so that even cells deeper in the culture have a near optimal exposure to light [122]. Overall, therefore small antenna cultures have a higher photosynthetic efficiency (Fig. 7b). In conclusion, small antenna mutants have the most advantages for biofuel production as follows: (1) Reduced heat losses based on fluorescence and LHCII (2) improved light penetration properties, (3) reduced photo-damage, (4) higher yield and improved bioreactor efficiency. The use of genetic engineered strains in conjunction with optimized mixing protocols may further enhance these efficiency gains.

Table 2 Profiles of fatty acids [92]

Fatty acid	Fatty acid	Cetane N°	Melting point (°C)	Ester m.p.
8:00	Caprylic	33.6	16.7	Ethyl, −43 °C
10:00	Capric	47.7	31.6	Ethyl, −20 °C
12:00	Lauric	61.4	44.2	Ethyl, −1.8 °C
14:00	Myristic	66.2	54.4	Ethyl, 12.3 °C
16:00	Palmitic	74.5	62.9	Ethyl, 24 °C
16:1ω7	Palmitoleic	45	−0.1	NA
18:00	Stearic	86.9	69.6	Methyl, 39 °C
18:1ω9	Oleic	55	14	Methyl, −20 °C
18:2ω6	Linoleic	36	−5	Methyl, −35 °C
18:3ω3	Linolenic	28	−11	Methyl, −57 °C
20:1ω9	Gadoleic	82	23	NA
20:4ω6	Arachidonic	NA	−50	NA

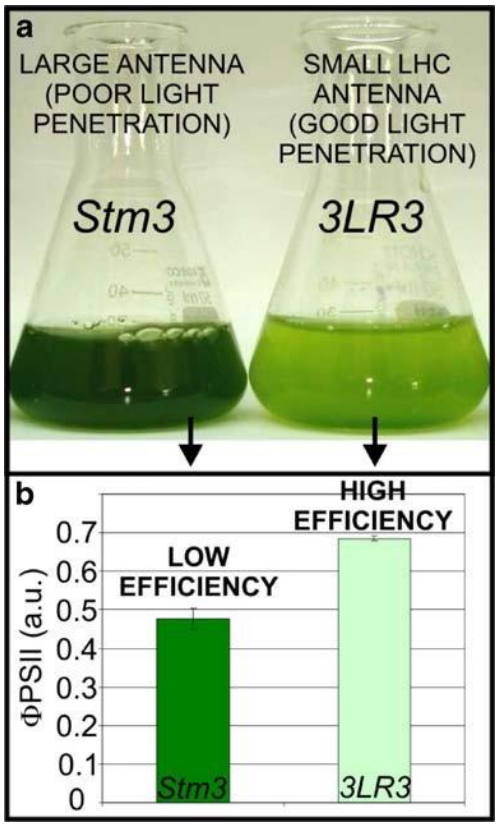


Fig.7. (a) Comparison of cultures of *Chlamydomonas reinhardtii* with parent strain (Stm3) and reduced antenna size (3LR3) at equal cell densities. a Cultures at densities of 6 × 106 cells/mL; (b) Photosynthetic quantum yield (8PSII); adapted from Mussgnug et al. [122]

Using Wastewater and Seawater Resources:

The use of wastewater and seawater offers clear advantages over placing increased pressure on freshwater resources (as water scarcity in the face of climate change and population growth is an increasingly important issue). However, both can vary in water quality, with wastewater varying dramatically from one source to another and also fluctuating over time. Wastewater can contain valuable nutrients such as nitrogen and phosphorous [10, 163], but can also contain

heavy metals, excessive trace metals, and other contaminants which are all of considerable concern— particularly in the context of biodiesel production, as heavy metals like cadmium have been reported to down-regulate lipid biosynthesis amongst many other cellular processes [64]. Seawater can also contain the same contaminants although rarely in the same concentrations. Furthermore, the use of cheaper agricultural grade fertilizers is economically desirable, but this represents another source of heavy metal contamination that can be inhibitory for sensitive strains of algae.

While conservation of freshwater resources is desirable, the selective advantage of strains that tolerate higher levels of certain contaminants can be of benefit in open pond systems and could theoretically help to address concerns of contamination by other opportunistic organisms. Significant advances have been made towards overcoming the problem of chemical contaminations, in some part due to the use of algae in phytoremediation and toxicology studies [206] which has also been the subject of substantial debate [15, 207]. Induction of oxidative stress is considered to be the underlying problem of many metal-related contamination issues [133, 171]. As well as heavy metals [62, 186–188, 197], trace metals like copper that are normally required for nutritional sufficiency, can also be inhibitory or lethal at excessive levels [22, 180], as can other non-metal organo- compounds [54]. The mechanisms of resistance are not only dependent upon algal species but also strains within particular species and obviously upon the type of toxin. While early studies [32, 33, 108] did not fully explain the resistance mechanisms, such as reduced accumulation, sequestration, and precipitation, more recent work has made significant progress in determination of potential protein [64] and gene targets [40] involved in heavy metal resistance in algae. Structural factors such as cell wall composition [61] and parallel studies on the resistance mechanisms in plant models [116] present areas for further research.

While many algal strains flagged for oil production are marine strains, others such as *Botryococcus braunii* are freshwater strains. Thus, the use of seawater for algal production can present osmotic problems in addition to those discussed above. Osmoadaptation in microorganisms has been extensively reviewed [60, 90]. Although salt stress has been shown to play a role in oil production [140, 176, 182, 183], freshwater species can undergo a stress response at osmolarities well below those of seawater, which can be strongly inhibitory or lethal. There have also been recent advances in this field with genes that exhibit anti-salt activity in microalgae [177]. Often these genes are generic anti-stress genes that likely act through alleviation of oxidative stress. A glutathione peroxidase-like protein from *Chlamydomonas* as reported by Yoshimura et al. [200] is one such example, which when cloned into tobacco induced an enhancement of stress tolerance including salt. Glutathione peroxidase has also been previously implicated in stress responses to heavy metal contamination [133]. Clearly strain selection and characterization in this context bear significant potential as well as the breeding, engineering, and adaptation of strains with desirable phenotypes that allow the use of water resources of varied water quality.

Recent Advances in Microalgal Production:

Recent advances were presented at the 1st International Algae Biomass Summit in San Francisco in November [3] which brought together the heads of newly-formed microalgae biofuel companies and existing aquaculture companies, many of the chief investigators from the Aquatic Species Program and microalgae scientists from around the world [3]. World experts were invited to collaborate and apply for significant funding opportunities in 2008 and beyond. The main aim was to develop a highly-efficient system for low- cost algal oil production and to optimize conversion to JP-8. The economic targets were given as algae hydrocarbon costs of US $0.48 per litre ($2 per gallon) with a minimum order of 210 million litres (50 million gallons). At this price, algae by-products would have to factor into the economics to make it viable. The invited speakers presented a combination of lessons from history, current technology and new ideas. Key findings and conclusions can be summarized as follows: Joseph C. Weissman calculated that the theoretical maximum possible yield for algal productions is 100 g m−2 day−1 or 365 tons dry biomass per hectare per year. Current limitations include that CO2 is not free at the high concentrations that are required for peak algal growth and that algal grazers are a significant, but a relatively ignored problem. Cost-effective harvesting has been and still is a major limiting factor. Biofuel production also reportedly requires biomass at a cost of less than $300 US/ton dry weight. Ami Ben-Amotz presented open pond yields averaging 20 g m−2 day−1 and that overall production costs of $0.34 US/kg are viable if the lipid content is high enough. A very cost-effective way for bioflocculation is the use of *Skeletonema* to co-bioflocculate high lipid *Nannochloropsis*. Tryg Lundquist reported that wastewater treatment ponds have a huge production potential and high nutrient contents and infrastructure are already in place. Limiting factors are the use of CO2 and harvesting. Mike Massingill demonstrated that harvesting of algae assisted by fish is very cost- effective. Mark Huntley presented hybrid systems growth rates of *Tetraselmis suecica* of 62 g m−2 day−1 with 30% lipid content, although not over a yearly average. Cheap bioreactors designs were presented by Bryan Willson using disposable plastic materials and Ben Cloud at a cost ~$15 US m−2 that have standard farm-style set ups.

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